SPECIFIC UNRESPONSIVENESS IN RATS WITH PROLONGED CARDIAC ALLOGRAFT SURVIVAL AFTER TREATMENT WITH CYCLOSPORINE

III. Further Characterization of the CD4+ Suppressor Cell and its Mechanisms of Action

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Peripheral T cells are divided into two subpopulations, $CD4^+$ cells that respond to antigen presented by class II MHC, and $CD8^+$ cells that respond to antigen presented by class I MHC (1). Studies of the allograft response demonstrate that both $CD4^+$ and $CD8^+$ subclasses of T cells can interact in the mediation of rejection (2–10). The relative roles of $CD4^+$ and $CD8^+$ cells in the rejection response has been established by adoptive reconstitution of T cell–depleted hosts with T cell subsets (2–8) and by treating hosts with mAbs to deplete one or both subsets (9). These studies show naive $CD4^+$ cells have a predominant role in the majority of strain combinations. Naive $CD8^+$ cells alone can effect rejection in some strains (7, 8), but are usually dependent upon help from $CD4^+$ cells (2–4). If cells are previously sensitized, $CD8^+$ cells alone can reconstitute rejection as they lose their dependence for help from $CD4^+$ cells (2, 6, 10). Ts cells also become activated during the allograft and other immune responses (11–13), particularly in circumstances where graft rejection is prevented by immunosuppressive therapy (12, 13) or enhancement (14, 15). For both rejection (2, 3, 7) and suppressor responses (14, 16, 17), it has been proposed that the class II MHC-reactive $CD4^+$ cells primarily act to induce the effector class I MHC-reactive $CD8^+$ subclass. In vitro studies show cell surface molecules, such as high and low molecular weight forms of T200, can be used to identify these effector and suppressor subpopulations of $CD4^+$ and $CD8^+$ T cells (16, 17).

We have examined the cellular basis of both allograft rejection (3, 10, 18) and the maintenance of specific unresponsiveness induced by cyclosporine (CSA)[1] treatment

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[1] *Abbreviations used in this paper:* AT_x, adult thymectomized; CSA, cyclosporine; DAB, Dulbecco's PBS with calcium and magnesium; LNC, lymph node cells; W/F, Wistar Furth.

(12, 13) in DA rats grafted with hearts from class I and II MHC-incompatible PVG donors. In this strain combination the restoration of graft rejection in sublethally irradiated hosts is dependent upon $CD4^+$ cells (3, 10, 18). Although naive $CD8^+$ cells alone do not effect rejection, synergy with $CD4^+$ cells has been demonstrated in that graft rejection time in hosts reconstituted with both $CD4^+$ and $CD8^+$ cells is faster than in those given $CD4^+$ cells alone (3). Specific sensitization does not alter the capacity of $CD4^+$ cells to restore rejection (10, 13), but sensitized $CD8^+$ cells show specific sensitization in that pure populations of these cells can restore graft rejection (10, 13). These results confirm the role of naive and sensitized $CD8^+$ cells as effectors of rejection that was proposed by in vitro studies of the alloimmune response (19, 20).

CSA or enhancing antibody treatment prevents rejection of organ allografts in rats and allows the development of specific unresponsiveness to donor strain grafts ~50 d after transplantation (12–15, 21–23). After this time $CD4^+$ cells from DA hosts are unable to restore original donor graft rejection, and can also inhibit the restoration of rejection by naive lymph node or $CD4^+$ cells (12, 13, 15). Before 50 d, $CD4^+$ cells retain the capacity to effect graft rejection, and $CD8^+$ alone gain the capacity to restore rejection in the irradiated host (13, 15), suggesting that they are sensitized to donor antigens.

In this study the nature of $CD4^+$ suppressor cells in DA rats with long-surviving PVG heart grafts was further characterized. Their capacity to block restoration of rejection by naive and sensitized $CD4^+$ and $CD8^+$ cells was examined to test whether this suppressor cell could mediate the downregulation of the $CD4^+$ cells and sensitized $CD8^+$ cells that are present in the host in the first few weeks before unresponsiveness develops. The requirement of $CD8^+$ cells for the mediation of suppression was also examined, in particular whether radioresistant or regenerating $CD8^+$ cells in the adoptive host were necessary for the reinduction of unresponsiveness. We also examined whether the $CD4^+$ suppressor cells could be subdivided from the $CD4^+$ cells that induce the rejection response by phenotype, by life span, and by sensitivity to cyclophosphamide (14, 16).

Materials and Methods

Rats. Rats of the DA (RT1a), PVG (RT1c), and Wistar Furth (W/F; RT1u) strain were bred and maintained in the Medical School Animal House, at the University of Sydney. In the U. S., DA and PVG were purchased from Bantin and Kingman (Fremont, CA) and W/F were from Harlan Sprague Dawley, Inc., (Indianapolis, IN).

Operative Procedures. DA rats, anesthetized with ether, were grafted with PVG or W/F hearts by end-to-side anastomosis of the ascending aorta to abdominal aorta and the pulmonary artery to the inferior vena cava, as described elsewhere (24). Donors were 150–200-g female rats, and recipients were 250–300-g male rats. Graft ischemia times ranged from 25 to 40 min, and all grafts commenced to contract within 2 min of reanastomosis. Graft function was monitored daily by palpation of contraction and electrocardiogram. On the day function was lost, graft tissue was obtained for histological confirmation of rejection. Experiments were terminated if grafts survived >100 d. Long surviving grafted hearts were removed by ligation of the aorta and pulmonary artery.

Thymectomy was performed, as described (25), on 4–6-wk-old DA rats, and animals were used 6 wk later.

Treatment of Rats to Induce Long-term Graft Survival. DA rats were grafted with PVG hearts and given CSA (Sandoz, Basle, Switzerland) at a dose of 20 mg/kg/d, for 10 d, as described (12). Grafts in treated animals survived for >100 d, compared with a survival time of 6–8 d

in untreated control animals. Unless otherwise stated, CSA-treated cell donors were animals who had PVG heart grafts functioning >75 d.

Cell Preparations. Single cell preparations from spleen and lymph node cells (LNC) were prepared in Dulbecco's PBS with calcium and magnesium (DAB; Oxoid, Oxford, UK) containing 10% FCS (Flow Laboratories, North Ryde, Australia), as described elsewhere (26). Subpopulations of lymphocytes were identified using an indirect immunofluorescence technique (12, 17), with the mouse mAbs MRC Ox19 (pan T, CD5) (27), W3/25 (CD4) (28), MRC Ox8 (CD8) (28), MRC Ox17 (Ia) (29), MRC Ox22 (CD45R) (30), and MRC Ox39 (IL-2-R) (31) (Sera Laboratories, Oxford, UK) as the first stage, and FITC-labeled rabbit anti-mouse Ig (Dako Corp., Copenhagen, Denmark) as the second stage. Ig^+ cells were identified using FITC-labeled rabbit anti-rat Ig (Dako Corp.) (26). Stained cells were enumerated on a FACS 440 or a FACS Star (Becton Dickinson & Co., Mountain View, CA).

Cell Separation. Full details of cell separation techniques have been described (18). Briefly, $CD4^+$ and $CD8^+$ T cell subsets were separated by binding MRC Ox8 or W3/25 at saturating concentrations to cells before panning on plastic petri dishes (Lab Tek; Miles Laboratories Inc., Naperville, IL) coated with both sheep anti-mouse Ig (Silneus Laboratories, Pty. Ltd., Hawthorn, Australia) and sheep anti-rat Ig (Silneus Laboratories, Pty. Ltd.). The nonadherent populations from MRC Ox8-bound cell populations was 90–95% W3/25$^+$, 1–2% Ig^+, and 1–4% MRC Ox8$^+$; that from W3/25 bound cell populations was 85–90% MRC Ox8$^+$, 1–4% Ig^+, and 2–8% W3/25$^+$. To separate subpopulations of $CD4^+$ cells, MRC Ox8 was combined with MRC Ox22, MRC Ox17, or MRC Ox39. Cells were immediately transferred, except in experiments examining survival in vitro, where they were cultured in RPMI (Flow Laboratories), 10% FCS, 5×10^{-5} M 2-ME, 2 mmol L-glutamine, penicillin (100 U/ml), and streptomycin (100 Ug/ml), in a 5% CO_2 incubator at 37°C.

Treatment with cyclophosphamide (20 mg/kg i.v.) (Bristol Laboratories, NSW, Australia) was given to cell donors 48 h before cell donation to study cell life span in vivo.

MCR Ox8 Treatment of Adoptive Host. MRC Ox8 was produced from the clone, generously provided by Dr. A. Williams, MRC Cellular Immunology Unit, Oxford, UK, in ascites in BALB/c mice (obtained from Radiation Biology, Stanford, CA) primed with IFA (Difco Laboratories, Inc., Detroit, MI). Ascites with 7 mg of MRC Ox8 was given intraperitoneally to the host two d before, then 1 and 4 d after grafting.

Adoptive Transferences. Donor and recipient rats for adoptive transfer experiments were given whole body irradiation; in Sydney at 1 Gy/min to a total dose of 9 Gy from a 60 Co source, as described before (26), and at Stanford at 1 Gy/min to a total dose of 6.25 Gy from a Philips X-ray machine (250 kV, 15 mA) (Philips Electronic Instruments, Inc., Mahwah, NJ). The dose of irradiation was determined as that required to prolong PVG and W/F skin graft survival from 8–10 d to > 15 d, but with a mortality of <20% from irradiation. Within 24 h the DA recipients were grafted with hearts from PVG or W/F donors. Irradiated DA recipients given no cells do not reject PVG or W/F hearts for at least 34 d, and many retain their grafts indefinitely (>150 d). The capacity to reject a heart from either strain can be restored to irradiated recipients by the adoptive transfer of normal syngeneic LNC or spleen cells, T cells, or W3/25$^+$ T cells (3, 18, 26). All subpopulations of cells from CSA-treated rats with long-surviving PVG allografts (>75 d) are unable to restore PVG, but do restore W/F heart graft rejection (12). W3/25$^+$, but not MRC Ox8$^+$ cells, are able to suppress the restoration of PVG heart graft rejection by naive LNC or W3/25$^+$ cells, when mixed in a ratio of 3–4:1 (12).

Statistics. Differences in rejection time between groups of rats restored with equivalent inocula were analyzed using the Wilcoxon rank sum test. A p value of <0.05 was considered significant.

Results

We have recently reported that during the induction phase, grafted rats destined to become unresponsive contain both W3/25$^+$ and MRC Ox8$^+$ cells with the capacity to restore donor strain rejection in irradiated adoptive hosts (13, 15) (see Table I). At 8 and 20 d post-grafting, the W3/25$^+$ T cell subset (data for day 8 shown

TABLE I

Comparison of Capacity of W3/25+ and MRC Ox8+ Cells from DA Rats Grafted with PVG Hearts, Either Treated or not Treated with CSA, to Adoptively Restore Rejection of PVG and W/F Heart Grafts in Irradiated DA Hosts

		Graft survival					
		PVG grafts			W/F grafts		
Cell donor	5 × 10^6 Cells	Median	Range	No.	Median	Range	No.
		d					
Naive	W3/25+	13	11-33	14	22.5	22-30	4
CSA-treated (day 8)	W3/25+	11	8-11	4	28	19-33	3
CSA-treated (day 7)	W3/25+	>100	>100	5*	21	21-26	5
No CSA (day 75)	W3/25+	19	11-24	6	35	19-43	8
Naive	MRC Ox8+	>100	>100	6	43	16-55	5
CSA-treated (day 8)	MRC Ox8+	52	6->100	13‡	28	16-47	8
No CSA (day 8)	MRC Ox8+	46	8->100	12‡	31	11->100	12
CSA-treated (day 75)	MRC Ox8+	>100	>100	4	47	14->100	3
No CSA (day 75)	MRC Ox8+	21	11-22	5§	>100	35->100	9
-	-	>100	40->100	8	>100	34->100	10

* Significantly different ($p < 0.001$) from the restorative capacity of all other W3/25+ populations tested.
‡ These groups had survival times intermediate between that of naive and sensitized rats, but did not reach significant differences with either.
§ Signifieantly different from naive and d75 CSA-treated cells ($p < 0.001$).

in Table I, line 2) cells retained the capacity to adoptively restore graft rejection of both PVG and W/F grafts, in a tempo comparable with that of naive cells. By day 50 and subsequently, the W3/25+ cells were unable to restore PVG rejection but could effect third party W/F rejection (data for day 75 shown in Table I, line 3). This change required both the maintenance of the graft and CSA treatment, as W3/25+ cells from rats grafted with PVG hearts but not treated with CSA did effect rejection of both PVG and W/F hearts in irradiated hosts (see Table I, line 4).

MRC Ox8+ cells from CSA-treated heart-grafted rats taken on days 8, 20, and 50 post-transplantation could restore PVG rejection in some adoptive hosts, but by 75 d and subsequently had lost their capacity to restore rejection (13) (data for day 8 and day >75 shown, Table I, lines 6 and 8). As naive MRC Ox8+ cells were unable to reconstitute PVG rejection (Table I, line 5) these findings showed that in the first 50 d there was activation akin to what occurs in unmodified rejection, in that MRC Ox8+ cells could effect rejection independent of W3/25+ cells (see Table I, line 7) (13). The sensitized state of MRC Ox8+ cells was not sustained, however. In sensitized hosts, 75-d post-transplant MRC Ox8 cells showed specific sensitization, in that they effected PVG but not W/F rejection (Table I, line 9) while day 75 MRC Ox8+ cells from CSA-treated rats with PVG grafts effected neither PVG nor W/F rejection (Table I, line 8). Thus, CSA treatment did not prevent a transient sensitization of MRC Ox8+ cells, nor did it immediately reduce the alloreactivity of W3/25+ cells. The W3/25+ cells lost alloreactivity and the MRC Ox8+ cells lost their sensitized state, ~50 d post-transplantation, which is about the time when unresponsiveness to second donor grafts is acquired by the CSA-treated host (22, 23). We have previously shown that W3/25+ (see Table II, line 1) and not MRC Ox8+

TABLE II

Capacity of W3/25⁺ Suppressor Cells to Inhibit Restoration of PVG Heart Graft Rejection by Naive or Sensitized W3/25⁺ Cells in Irradiated DA Rats

Source of 5 × 10^6 W3/25⁺ cells DA sensitized:		Rejection time					
		Hearts surviving with suppressor cells*			Hearts surviving without suppressor cells		
To strain	On day	Median	Until day (n)	p	Median	Until day (n)	
-	0	>100	14, 15, 17, >100(7)	<0.01	13	11(2), 12(5), 14, 18(5), 33	
PVG	8	22	15, 18, 25, 53	NS	10	10(3), 22(2)	
PVG	75	15	15(4)	NS	24	11, 13, 24(3)	
W/F	8	>100	30, >100(4)	<0.05	14	11, 14(2)	
PVG/Rx CSA	8	>30	15, >100(4)	<0.05	11	8, 11(3)	

* 2 × 10^7 W3/25⁺ suppressor cells from CSA-treated DA rats with PVG heart grafts.

cells from DA rats with PVG hearts surviving >75 d could suppress the reconstitution of rejection in irradiated hosts of PVG, but not third party hearts by naive W3/25⁺ cells (12). As both the CD4 and CD8 cells in CSA-treated hosts appear to be sensitized to PVG shortly after transplantation, we examined whether the W3/25⁺ suppressor cells in rats with long surviving grafts could account for the downregulation of these sensitized cells' capacity to effect rejection.

Examination of the Capacity of W3/25⁺ Cells from Unresponsive Rats to Suppress Specifically Sensitized W3/25⁺ cells. The effect of W3/25⁺ cells from CSA-treated rats on the various population of W3/25⁺ cells is shown in the left hand column of Table II and the capacity to effect rejection of the W3/25⁺ cells alone is shown in the right hand column of Table II. W3/25⁺ cells from CSA-treated rats suppressed 5 × 10^6 naive W3/25⁺ cells (Table II, line 1) but did not suppress sensitized W3/25⁺ cells taken either 8 (line 2) or 75 d (line 3) after grafting. Specifically, sensitized W3/25⁺ (5 × 10^6) cells obtained from DA rats 8 and 75 d after grafting a PVG heart restored rejection in a tempo similar to that of equivalent numbers of naive W3/25⁺ cells (see Table II, right hand column). This is the first difference we were able to demonstrate between naive and sensitized W3/25⁺ cells in the alloimmune response of DA rats. This effect of sensitization was shown to be specific; in that W3/25⁺ cells from CSA-treated rats did suppress W3/25⁺ from animals that had rejected a third-party heart (Table II, line 4).

Even though suppressor W3/25⁺ cells from CSA-treated rats did not suppress day 8 specifically sensitized W3/25⁺ cells, they did suppress the restoration of PVG rejection by W3/25⁺ cells from day 8 CSA-treated DA rats grafted with PVG hearts (Table II, line 5). These results suggest that CSA may prevent or limit the sensitization of W3/25⁺ cells during the induction phase. It was concluded that the W3/25⁺ suppressor cell from specifically unresponsive rats impaired the capacity of the W3/25⁺ cells present in the CSA-treated host for several weeks after transplantation to effect rejection, even though it was unable to suppress rejection by sensitized W3/25⁺ cells.

Examination of the Capacity of W3/25⁺ Cells from Unresponsive Rats to Suppress MRC Ox8⁺ Cells. Naive MRC Ox8⁺ cells alone did not adoptively restore rejection in irradiated hosts (3); therefore, the effects with W3/25⁺ suppressors on these cells could not be examined without the addition of naive W3/25⁺ cells (Table III, line

TABLE III

Capacity of W3/25+ Suppressor Cells to Inhibit Restoration of PVG Heart Graft Rejection by Normal or Sensitized MRC Ox8+ Cells in Irradiated DA Rats

Restorative inocula			Rejection time					
			With suppressor cells*			Without suppressor cells		
MRC Ox8+		No. naive		Hearts surviving			Hearts surviving	
No.	Sensitized to	W3/25+	Median	until day (n)	p	Median	until day (n)	
2×10^7	No	–	–	ND	–	>100	73, >100(4)	
2×10^7	No	5×10^6	24.5	16(4), 35(2), >100(2)	<0.01	7	7(3), 9(2)	
5×10^6	PVG d8	–	>100	18, >100(3)	NS	40	8, 12, 17(3), 40, 51, 54, 66(2), >100(2)	
5×10^6	PVG d>75	–	>100	26, >100(4)	<0.05	21	21(4), 22	

* 2×10^7 W3/25+ Suppressor cells from CSA-treated DA rats with PVG heart grafts.

1). In rats restored with both 2×10^7 MRC Ox8+ and 5×10^6 naive W3/25+ cells (Table III, line 2), rejection occurred faster than in rats restored with naive 5×10^6 W3/25+ cells alone (Table II, line 1). 2×10^7 W3/25+ cells from CSA-treated rats significantly delayed rejection in rats restored with both the 5×10^6 naive W3/25+ and naive 2×10^7 MRC Ox8+ cells and induced indefinite survival in two of eight rats (Table III, line 2). This inhibitory effect of W3/25+ suppressors was not as great as that against 5×10^6 naive W3/25+ cells alone, where 7 of 10 rats went on to indefinite graft survival (Table II, line 1).

Sensitized MRC Ox8+ cells were also suppressed by W3/25+ cells from CSA-treated hosts. MRC Ox8+ cells taken 8 or 75 d post-grafting from normal rats that had rejected a PVG graft were able to restore rejection without help from naive W3/25+ cells (Table III, lines 3 and 4). MRC Ox8+ cells taken at day 8 did not invariably restore rejection, but those taken at day 75 did. There appeared to be suppression by the W3/25+ CSA cells of both day 8 and 75 sensitized MRC Ox8+ cells, although due to the variable capacity of day 8 MRC Ox8+ cells alone to restore rejection, the effect of the W3/25+ suppressor cell did not reach significance. These findings showed that the W3/25+ suppressor cell could directly inhibit the generation and/or action of sensitized MRC Ox8+ cells as effectors of rejection. Thus, the W3/25+ suppressor could account for the inhibition of the sensitized MRC Ox8+ cells that occurs in CSA-treated hosts during the first 2 mo post-transplant. The greater proportion of rejected grafts in the group given naive MRC Ox8+ cells suggests that rather than acting as effectors of suppression, the cells were activated as effectors of rejection. These experiments did not examine the role adoptive host-derived MRC Ox8+ cells might play as effectors of suppression, however.

Examination of the Role of Adoptive Host-derived MRC Ox8+ Cells in the Mediation of Suppression by W3/25+ Cells. The role that host-derived MRC Ox8+ cells may play as effectors of suppression that are activated by the transferred W3/25+ suppressor cells were examined using adult thymectomized (AT_x) irradiated hosts in which regeneration of MRC Ox8+ T cells is limited (Table IV), or by treating the adoptive hosts with MRC Ox8 mAb to deplete these hosts of radioresistant MRC Ox8+ cells (Table V).

TABLE IV

Capacity of W3/25⁺ Suppressor Cells to Inhibit Restoration of PVG Hearts by Naive W3/25⁺ Cells in Irradiated DA Rats Further Depleted of MRC Ox8⁺ Cells by Thymectomy

Restorative inocula		Irradiated host treatment	Rejection time		
No. suppressor W3/25⁺*	No. naive W3/25⁺		Median	Hearts surviving until day (n)	p
2×10^7	5×10^6	–	>100	14, 15, 17, >100(7)	–
2×10^7	5×10^6	AT_x[‡]	>100	>100(5)	NS
–	5×10^6	AT_x[‡]	27	17, 20, 27, 31, >100	<0.001
–	5×10^6	–	13	11(2), 12(5), 14, 18(5), 33	NS
–	–	–	>100	40, 53, 68, >100(5)	<0.001

* W3/255⁺ suppressor cells from CSA-treated rats with PVG heart grafts.
‡ AT_x recipient DA rats were thymectomized 6 wk before grafting.

In a previous study, it was shown that whole body irradiation depletes MRC Ox8⁺ and W3/25⁺ T cell populations to <5% of normal peripheral lymphoid tissue content (25). Regeneration starts within 2 wk and CD8⁺ cells recover more rapidly than CD4⁺ cells. Both subgroups are repleted to near normal by 7 wk. In AT_x irradiated hosts, recovery of CD8⁺ T cells is nearly abolished and there is only a 10% recovery of CD4⁺ T cells (25).

Thus, we examined whether cells produced by the regenerating thymus of the irradiated adoptive hosts would be required to contribute to reestablishment of unresponsiveness. This was not the case, as W3/25⁺ cells from unresponsive hosts suppressed the restoration of graft rejection by naive W3/25⁺ cells in irradiated AT_x hosts in all five rats tested (Table IV, line 2), compared with 7 of 10 in non-AT_x hosts. AT_x did not prevent naive W3/25⁺ cell restoring rejection, as irradiated AT_x con-

TABLE V

Examination of the Effect of Depletion of MRC Ox8⁺ Cells from Irradiated DA Hosts by Treatment with mAb MRC Ox8 on the Transfer by W3/25⁺ Suppressor Cells of Unresponsiveness Toward PVG Heart Grafts

Restorative inocula: no. W3/25⁺ cells		Rejection time				
		Irradiated hosts		Irradiated and MRC Ox8-treated hosts		
Suppressor*	Naive	Median	Heart surviving until day (n)	Median	Heart surviving until day (n)	Significance (p)
–	–	>100	>100(4)	16.5	13, 16, 17, 39	0.05
						NS
2×10^7	–	>100	>100(5)	38	36(2), 38, 40, >40	0.05
						0.01
2×10^7	5×10^6	>100	>100(4)	11	7(2), 11, 13, 15	0.05
						NS
–	5×10^6	13	13(4)	14	10, 11, 14(2), 20	NS

* W3/25⁺ suppressor cells from CSA-treated DA rats with PVG heartgrafts.

trols restored with naive W3/25+ cells alone rejected their grafts (Table IV, compare lines 3 and 4), albeit with a trend to a slower tempo in AT_x hosts. This latter result confirms that the effect of W3/25+ suppressors was inhibitory and that the AT_x host when reconstituted with W3/25+ cells did not lack cells necessary to reject the graft. Taken together, these results strongly suggest that thymus-derived cells are not required to be effectors of the suppressor response transferred by the W3/25+ cells.

As irradiation does not totally ablate MRC Ox8+ cells in peripheral lymphoid tissues (25), we examined the role these cells may play in transfer of suppression, by treating the adoptive host with the mAb MRC Ox8. This antibody is effective at nearly totally depleting MRC Ox8+ cells in normal hosts (32); and in irradiated hosts, no MRC Ox8+ cells can be found in lymph node or spleen 2 wk after treatment with both irradiation and MRC Ox8 therapy (Gurley, K. E. and B. M. Hall, unpublished results). Surprisingly, controls treated with MRC Ox8 and irradiation, but not restored with any cells, all rejected their grafts, while irradiated rats given neither MRC Ox8 nor cells did not reject their grafts (Table V, line 1). This suggested that MRC Ox8+ cells in irradiated hosts suppressed recovery of the alloimmune system and prevented rejection of the graft. Irradiated MRC Ox8-treated hosts given W3/25+ suppressor cells from CSA-treated rats also rejected their grafts, while non-MRC Ox8-treated hosts never rejected (Table V, line 2). Although this tended to be slower than nonreconstituted irradiated, MRC Ox8-treated controls, the difference did not reach significance. Further, W3/25+ suppressor cells, mixed with naive W3/25+ cells, failed to inhibit the latter cells in irradiated MRC Ox8-treated hosts (Table V, line 3); whereas in non-MRC Ox8-treated irradiated hosts, the same naive cells are suppressed. MRC Ox8 treatment of irradiated hosts restored with naive W3/25+ cells did not impair these cells' capacity to affect rejection, in that the rejection tempo was similar to that in irradiated hosts restored with only naive W3/25+ cells (Table V, line 4).

Taken together, these results show the rejection response in irradiated rats is inhibited by an MRC Ox8+ cell that was radioresistant but not thymus derived. This cell was critical for the transfer of suppression by the W3/25+ cells from CSA-treated hosts.

Role of Thymus and MRC Ox8 Therapy in Generation of W3/25+ Suppressor Cells in CSA-treated Rats. The independence of the generation and maintenance of suppression from the thymocytes or cells recently derived from the thymus was further confirmed by experiments in which unresponsiveness was induced by CSA in AT_x hosts. Indefinite graft survival was induced in all AT_x hosts treated with CSA ($n = 10$), and in control AT_x rats not treated with CSA, all grafts were rejected in first set (7 d [$n = 6$]). 2×10^7 W3/25+ cells taken from these AT_x CSA-treated rats with long surviving grafts (>75d) suppressed the capacity of 5×10^6 naive W3/25+ cells to effect rejection in the adoptive transfer assay, all four grafts surviving >100 d (Table VI, line 2). W3/25+ cells from AT_x donors that had not been grafted or treated with CSA restored graft rejection in a normal tempo (Table VI, line 3), which indicated that thymectomy itself did not result in CD4+ cells becoming suppressor cells or in the depletion of these cells. It was concluded that cells recently derived from the thymus did not contribute to the generation of suppressor cells. This complements the finding that reestablishment of unresponsiveness in the irradiated adoptive host

TABLE VI

Capacity of W3/25⁺ Cells from Thymectomized CSA-treated DA Rats with PVG Hearts to Restore PVG Heart Graft Rejection in Irradiated DA Rats

Restorative inocula			Rejection time		
No. suppressor W3/25	No. naive W3/25	Donor/s AT,*	Median	Cell hearts surviving until day (*n*)	*p*
2×10^7	5×10^6	–	>100	14, 15, 17, >100(7)	NS
2×10^7	5×10^6	+	>100	>100(4)	<0.01
–	5×10^6	+	24	9, 16, 24, 26(2)	NS
–	5×10^6	–	13	11(2), 12(5), 14, 18(5), 33	

* Adult rats were thymectomized 6 wk before grafting.

was also thymus independent. Treatment of normal hosts with CSA and MRC Ox8 for 2 wk also did not impair induction of unresponsiveness. MRC Ox8 therapy to normal DA hosts had only minor effects in delaying PVG rejection from 6–8 to 8–10 d, even though this therapy depleted peripheral $CD8^+$ cells to <2% of peripheral blood lymphocytes compared with 15–18% in normals.

Phenotype of the W3/25⁺ Suppressor Cell. Using the panning technique, $W3/25^+$ suppressor cells were further depleted of cells identified by the mAbs MRC Ox17, MRC Ox22, and MRC Ox39. MRC Ox17, which identifies Ia antigens of the rat, was found on 12% of $W3/25^+$ cells from both naive and CSA-treated rats with long surviving grafts. Depletion of these cells did not totally eliminate suppression, although $W3/25^+$ MRC $Ox17^-$ cells did not appear as effective as the unfractionated $W3/25^+$ cells (Table VII, line 1). In all rats there was a rejection episode in the 14–30-d post-transplant period, from which four of eight grafts recovered function. Two of the rats that recovered subsequently rejected their grafts at 53 d. These rejection crises were never observed in rats restored with unfractionated $W3/25^+$ cells from unresponsive hosts. MRC Ox22 binds to 50–60% of $W3/25^+$ cells in CSA-treated and naive rats, and removal of this subset depleted suppressor function (Table VII, line 2).

Removal of the IL-2-R-bearing cells by MRC Ox39 therapy also abolished the

TABLE VII

Examination of the Phenotype of W3/25⁺ Suppressor Cells to Inhibit Adoptive Restoration of PVG Heart Graft Rejection by Naive W3/25⁺ Cells

Restorative inocula		Rejection time		
2×10^7 W3/25⁺ suppressor cells* depleted with the mAb	No. naive W3/25	Median	Hearts surviving until day (*n*)	Significance‡
MRC Ox17 (Ia)	5×10^6	43	16, 20, 33(2), 53(2), >100(2)	$p < 0.001$
MRC Ox22 (CD45R)	5×10^6	21	14, 18, 21, >100	NS
MRC Ox39 (IL-2-R)	5×10^6	15	14(2), 15, 39, >100	NS
Nil	5×10^6	>100	14, 15, 17, >100(7)	$p < 0.01$
–	5×10^6	13	11(2), 12(5), 14, 18(5), 33	–

* W3/25⁺ suppressor cells from CSA-treated DA rats with PVG heart grafts.

‡ Significance compared with rats restored with 5×10^6 naive W3/25⁺ cells alone.

suppressive capacities of the W3/25+ cells (Table VII, line 3). In CSA-treated animals and normal DA rats, IL-2-R+ cells accounted for 9 and 5%, respectively, of the W3/25+ population.

Life Span of W3/25+ Suppressor Cells. The life span of the W3/25+ suppressor cell was assessed in several ways. Its dependence on antigen in the functioning graft was tested by removal of the heart and transferring the W3/25+ cells 1 and 3 wk after the heart graft was removed. By 8 d after removal of the heart graft, W3/25+ cells from CSA-treated rats (Table VIII, compare lines 1 and 2), which were 50 d post-grafting, restored rejection in three of four rats. In contrast, W3/25+ cells from controls that had not had their grafts removed were unable to effect rejection in three of four adoptive hosts. W3/25+ taken 11–21 d after removal of the heart in CSA-treated rats that had maintained a PVG graft for >100 d did not effect rejection in four of six rats (Table VIII, compare lines 3 and 4).

Cyclophosphamide given to unresponsive CSA-treated hosts, 48 h before adoptive transfer of W3/25+ cells, abolished their capacity to suppress in all four rats tested (Table VIII, line 6), compared with those from noncyclophosphamide-treated hosts, which suppressed in 7 of 10 (Table VIII, line 7). Cells from cyclophosphamide-treated unresponsive animals on their own were unable to restore rejection (Table VIII, line 5). The latter was not an effect of cyclophophamide, as W3/25+ cells from naive rats given cyclosphosphamide retained normal capacity to effect rejection (data not shown). This result suggested cyclophosphamide partially depletes suppressor cells.

Cells from CSA-treated rats cultured in vivo for 3 d (Table VIII, line 9), but not 1 d (Table VIII, line 8), regained the capacity to restore rejection and, by inference, lost the capacity to suppress.

These experiments showed that the W3/25+ suppressor cell had a short lifespan and was probably dependent on cell division and exposure to graft antigens. After cyclophosphamide treatment the W3/25+ cells' capacity to suppress naive cells preceded the recovery of their capacity to effect rejection. However, in vitro the population of cells that survived 3 d in culture did have the capacity to effect rejection. This suggested that there were two populations within the W3/25+ cells of CSA-treated hosts unresponsive to allografts, a short-lived suppressor and a longer lived cell with the potential to effect rejection. If this is the case, the short-lived suppressor cells prevented the mediation of graft destruction by the longer lived W3/25+ cells with the potential to effect rejection.

Discussion

Our studies demonstrated that transfer of specific suppression was dependent upon the CD4+ (W3/25+) cells alone and that this cell had the capacity to induce the unresponsive state in the CSA-treated host, in that it was capable of inducing the down-regulation of the normal alloreactive CD4+ T cells and specifically activated CD8+ cells that were present in the host in the first weeks after transplantation. The reestablishment of unresponsiveness in adoptive hosts by CD4+ suppressor cells was dependent upon a radioresistant MRC Ox8+ cell, which was not depleted by irradiation and thymectomy. Depletion of this MRC Ox8+ cell required MRC Ox8 antibody therapy in addition to irradiation. The nature of this MRC Ox8+ cell, whether it be a T cell or a natural suppressor cell, is yet to be defined, as is its mecha-

TABLE VIII

Experiments to Examine the Life Span of W3/25⁺ Suppressor Cells Using the Irradiated Adoptive Host to Examine for W3/25⁺ Cell Function

Restorative inocula				Rejection time		
W3/25⁺ cells from CSA-treated rats*			5×10^6 W3/25⁺ from naive DA	Hearts surviving until day		p
Treatment of cell donor or cells	d Post-transplant	No.		Median		
–	~50	5×10^6	–	>100	36, >100 (3)‡	NS
Graft removed 8 d earlier	~50	5×10^6	–	20	11, 15, 25, >100	
–	~100	5×10^6	–	>100	>100 (6)	NS
Graft removed 11–21 d earlier	~100	5×10^6	–	>100	17, 20, >100 (4)	
Cyclophosphamide 2 d earlier	>75	2×10^7		>100	41, 75, 78, >100 (4)	<0.001
Cyclophosphamide 2 d earlier	>75	2×10^7	+	25	16, 22, 26 (2)	NS
–	>75	2×10^7	+	>100	14, 15, 17, >100 (7)	
Cultured in vitro 1 d	>75	5×10^6	–	>100	>100 (8)	<0.001
Cultured in vitro 3 d	>75	5×10^6	–	19	6, 18, 20, 21	
–	>75	5×10^6	–	>100	>100 (6)	<0.01

* W3/25⁺ suppressor cells from CSA-treated DA rats with long-surviving PVG allografts.
‡ Numbers in parentheses are numbers of hearts surviving to that day.

nism of collaboration with specific CD4⁺ suppressor cells to promote reestablishment of the unresponsive state in the irradiated host. The role of these cells in the maintenance of unresponsiveness was unclear, as extensive studies in this model have failed to show that CD8⁺ cells alone can transfer unresponsiveness (22); if they are suppressor, their function may be dependent upon the CD4⁺ suppressor. Their role in the induction of unresponsiveness by CSA is also unclear, as MRC Ox8 therapy combined with CSA did not prevent induction of unresponsiveness. We also demonstrated that CD4⁺ suppressor cells expressed CD45R, IL-2-R, and probably class II MHC. CD4⁺ suppressors were not dependent upon cells recently derived from the thymus, but were relatively short lived and dependent upon the graft, presumably as a source of alloantigen to prime the response. This CD4⁺ suppressor cell had many characteristics of the suppressor/inducer cell described in other models (11, 14, 16, 33, 34).

Suppression primarily mediated by CD4⁺ cells occurs in neonatally induced tolerance to alloantigen (35), in a tumor allograft model (36, 37), and in active and passive enhancement models (15, 33). The development of a CD4⁺ suppressor after the activation of CD8⁺ cytotoxic effector cells is also similar to the findings of North and Bursuker (36, 37), who show that after transplantation of tumor grafts into semisyngeneic hosts, Ly-1⁻, -2⁺ sensitized effector cells are generated but fail to destroy the tumor because they are downregulated by Ly-1⁺, -2⁻, Ts cells. Similarly, in CSA-treated hosts and other models that go on to develop specific unresponsiveness, specific

cytolytic T cells can be isolated from the host shortly after transplantation (38, 39), but do not effect irreversible graft destruction, perhaps due to their inhibition by the suppressor cells. Our studies have shown that $CD8^+$ cells in CSA-treated hosts become sensitized to graft antigens, but this sensitization is lost after 50 d, a time when specific unresponsiveness is manifest (13). In this study we directly demonstrated that the $CD4^+$ suppressor cell could inhibit the capacity of sensitized $CD8^+$ cells to effect graft rejection; thus demonstrating that the suppressor cell had the capacity to switch off this arm of the rejection response. With $CD4^+$ cells, the suppressors inhibited naive $CD4^+$ cells as well as $CD4^+$ cells from CSA-treated hosts at a time shortly after transplant when they had the capacity to effect rejection. The $CD4^+$ suppressor's inability to inhibit sensitized $CD4^+$ cells was the first difference between sensitized and naive $CD4^+$ cells that we have demonstrated. A similar failure to inhibit sensitized cells but not naive cells has been observed in experiments using unfractionated cells from rats with specific unresponsiveness induced by antithymocyte globulin (40). Further, cells from rats with specific unresponsiveness to their allograft induced by either CSA or enhancement protocols only delay the restoration of rejection by sensitized $CD4^+$ cells in AT_x-irradiated and bone marrow-reconstituted rats, and do not reinduce unresponsiveness (4, 33, 41, 42). The failure of Ts cells to inhibit sensitized cells may also explain the difficulty in inducing unresponsiveness in specifically sensitized hosts (43, 44).

The finding that the $CD4^+$ suppressor is MRC $Ox22^+$ (CD45R) is consistent with previous reports that the suppressor/inducer cells in rats (30, 35) and man (45) express CD45R, the high molecular weight form of T200. This marker does not differentiate the suppressor cell from the effectors of rejection, in that the $CD4^+$ MRC $Ox22^+$ population in rats contains the T cells that produce IL-2 (46), which proliferate to alloantigen in vitro and in graft vs. host disease (30), and which in naive hosts reconstitute rejection to irradiated hosts (Gurley, K. E. and B. M. Hall, unpublished data). As memory cells that provide help for antibody responses to soluble antigen are MRC $Ox22^-$ (30, 46), it is unlikely that the MRC $Ox22^+$ suppressor cell mediates its effect by induction of an alloantibody response, akin to enhancement. This mechanism is also unlikely because of our previous findings that irradiation markedly depleted B cells (25, 26), and that transfer of $W3/25^+$ T cells from naive or unresponsive hosts did not restore in alloantibody production, unless large numbers (10^6) of B cells were also given (26). It has been proposed that $CD45R^+$ cells are naive cells, and that activated and memory cells cease to express the phenotype and express the low molecular weight form of T200 (47, 48). If this is the case, the suppressor cell fails to make this switch in T200, and retains the naive T cell phenotype.

The cells required to produce $CD4^+$ Ts cells were shown not to be recently derived from the thymus, making it likely they are derived from long-lived peripheral $CD4^+$ cells. Once induced, the $CD4^+$ suppressor cells appeared to have a short life span, and their sensitivity to cyclophosphamide suggested they were dividing. Their expression of an IL-2-R suggested these cells required IL-2 and perhaps other cytokines to sustain their survival. Preliminary studies have suggested suppressor cells can be sustained on cultures supplemented with cytokines (Pearce, N. W., K. E. Gurley, and B. M. Hall, manuscript in preparation). After removal of the graft, the recovery of the $CD4^+$ populations' capacity to effect rejection was slower the longer the graft

was in place. This finding is consistent with the strengthening in the degree of unresponsiveness with time as manifest by the lengthening in time to rejection of donor strain grafts (22, 23, 43).

The precise role of $CD8^+$ cells in the mediation of suppression was not directly resolved by these studies. We have previously shown that $CD8^+$ cells from CSA-treated hosts lack the capacity to transfer suppression and, in this study, showed that the normal $CD8^+$ cell population in peripheral lymphoid tissue could be suppressed by $CD4^+$, but did not augment induction of suppression. Further, the sensitized $CD8^+$ cell's capacity to affect rejection could also be abrogated by $CD4^+$ cells. The irradiated hosts, however, had radioresistant $CD8^+$ cells in which suppressor function predominated. This result was in contrast to Roser (35), who found that transfer of neonatal tolerance is not affected by MRC Ox8 treatment of the irradiated host. In our studies MRC Ox8 treatment of irradiated hosts to deplete them of $CD8^+$ cells allowed the $CD4^+$ cells within the host to effect rejection, thus abrogating the potential of irradiation to induce an unresponsive state. In a detailed examination of this effect (Gurley, K. E. and B. M. Hall, manuscript in preparation), we have demonstrated that the mediation of rejection in irradiated MRC Ox8-treated hosts is dependent on $CD4^+$ cells regenerated by the thymus, and that the MRC Ox8-treated hosts remain devoid of $CD8^+$ cells for several weeks. Given these observations, the failure of the $CD4^+$ suppressor cells to establish unresponsiveness in MRC Ox8-depleted irradiated hosts may have been due to an unfavorable shift in the balance of host lymphoid cells to the effector cascade. We have shown that the transfer of suppression was dependent upon a suppressor to effector ratio of 2–4:1, and that with a smaller ratio suppression is not transferred (Hall, B. M., unpublished data). In this study, several findings suggested the unresponsive hosts' $CD4^+$ cells were also not depleted of cells with the potential to effect rejection, particularly the loss of suppression after 3 d in culture, which is associated with a re-emergence of $CD4^+$ cells with the capacity to effect rejection. The failure of $CD4^+$ suppressors to prevent rejection in MRC Ox8-treated irradiated hosts suggested that the radioresistant MRC $Ox8^+$ cells were critical for the reestablishment of suppression. The MRC $Ox8^+$ cells that facilitate reestablishment of suppression were not derived from regenerating thymus, however. Whether these MRC Ox8 cells are T cells or natural suppressor cells similar to those described by Strober (49) requires further studies, as does their role in the reestablishment of suppression; in particular, whether they acquire specificity.

In nonalloimmune in vivo systems (11, 50) and alloimmune in vitro (16) and in vivo (14) studies, a network of cells involved in the induction and expression of tolerance has been demonstrated. These studies conclude that $CD4^+$ cells act as inducers and transducers of the suppressor response. The $CD4^+$ cells respond to the immunizing antigen and mediate their suppressive effect by activating $CD8^+$ suppressor effector cells that react to idiotype and are the mediator of suppression by directly impairing the effector arm of the immune response. In our experiments, we have not yet elicited what role the $CD8^+$ cells play in the mediation of suppression. The $CD8^+$ cells involved in the reinduction of unresponsiveness are not predominate in mature peripheral T cells, and their role as an effector of suppression has not been established, only a role in facilitating reinduction of unresponsiveness. Our studies suggest that if MRC $Ox8^+$ cells are required for the maintenance of

unresponsiveness, they must be highly dependent upon the CD4+ suppressor. One possibility not excluded by these experiments is that in mature peripheral lymphoid cell populations, there is a fine balance between CD8+ cells that are cytotoxic T cell precursors and those that have suppressor effector potential. As these two populations can be subdivided in man by the mAb 9.3 (CD28) (51), if an equivalent reagent was available in the rat, cytotoxic precursor cells could be depleted so the function of the CD8+ suppressor subset could be directly assessed.

While these studies have further characterized the suppressor response that mediates unresponsiveness in this strain combination, several important questions remain to be answered. These include how the transferred CD4+ suppressor and host radioresistant CD8+ cells mediate inhibition of CD4+ and CD8+ effector cells. The specificity of the CD4+ suppressor cells' receptor, whether it be idiotypic or antiidiotypic, also remains to be determined, as does its interaction with the radioresistant CD8+ cell in the adoptive hosts and the specificity of this subpopulation. It is possible that this cell recognizes donor alloantigen and prevents effective function of CD4+ and CD8+ cells by release of negative regulatory cytokines, or it may directly interact with these effectors via idiotype. These possibilities are currently under investigation, as is the best method to propagate the cell in vitro and to detect its presence by an in vitro assay. The understanding of the mechanisms of this form of unresponsiveness to allograft has potential for clinical transplantation, where its induction may allow minimization of maintenance immunosuppressive drugs.

Summary

The cellular basis of the specific unresponsiveness that develops in DA rats treated with cyclosporine (CSA) for 10 d after grafting a PVG heart was examined using an adoptive transfer assay. CD4+ cells from rats with long survival grafts specifically lack the capacity to restore PVG heart graft rejection, and can also inhibit the capacity of naive T cells to restore rejection, while in the first few weeks post-transplant, both CD4+ and CD8+ T cells from CSA-treated hosts have the capacity to effect PVG graft rejection. In this study, we demonstrated the CD4+ suppressor cells also had the capacity to inhibit restoration of rejection by CD4+ cells from CSA-treated DA rats recently transplanted with PVG hearts, and from rats sensitized to third party, but not from those specifically sensitized to PVG. They also inhibited the capacity of both naive CD8+ and sensitized CD8+ cells to effect rejection. These results showed that the CD4+ suppressor cell was capable of overriding the capacity to effect rejection of the CD4+ cell and activated CD8+ cells that were present in the CSA-treated host shortly after transplantation. The failure of naive CD8+ cells to augment suppression and the capacity of CD4+ suppressor cells to transfer unresponsiveness to irradiated hosts in which regeneration of CD8+ cells was abolished by thymectomy suggested that it was the CD4+ cell alone that mediated suppression. However, the failure of CD4+ suppressor cells to reinduce unresponsiveness in irradiated hosts whose CD8+ cells had been depleted by therapy with the mAb MRC Ox8 showed that a radioresistant CD8+ cell was required to reestablish the state of specific unresponsiveness. The induction of CD4+ suppressor cells in thymectomized hosts suggested that these cells were derived from long-lived CD4+ lymphocytes. However, their sensitivity to cyclophosphamide and their loss of suppressor function both after removal of the graft and after 3 d in culture demonstrated that

the suppressor cell itself had a short lifespan. The $CD4^+$ suppressor was shown to be MRC $Ox22^+$ ($CD45R^+$), MRC $Ox17^+$ (MHC class II), and MRC $Ox39^+$ (CD25, IL-2-R). These studies demonstrated the $CD4^+$ suppressive cell identified in rats with specific unresponsiveness induced by CSA therapy had many features of the suppressor inducer cell identified in in vitro studies of the alloimmune response. The $CD4^+$ suppressor cell could account for the downregulation of the $CD4^+$ and $CD8^+$ T cells, with the capacity to effect rejection that was present in the CSA-treated hosts shortly after transplantation, and its action was facilitated by a radioresistant $CD8^+$ cell that did not appear to predominate in mature peripheral $CD8^+$ T cells.

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